Breast Cancer Res Treat (2009) 116:53–68
DOI 10.1007/s10549-008-0081-7

PRECLINICAL STUDY

The prognostic significance of Ki67 before and after neoadjuvant chemotherapy in breast cancer

Robin L. Jones · Janine Salter · Roger A'Hern · Ash Nerurkar · Marina Parton · Jorge S. Reis-Filho · Ian E. Smith · Mitchell Dowsett

Received: 21 May 2008 / Accepted: 22 May 2008 / Published online: 1 July 2008

Abstract *Purpose* To compare the prognostic significance of proliferation, as assessed by Ki67 expression, in breast cancer before and after neoadjuvant chemotherapy. *Methods* A retrospective search of a prospectively maintained clinical database was performed to identify patients treated with neoadjuvant chemotherapy at the Royal Marsden Hospital. The expression of Ki67 was assessed using immunohistochemistry in pre-therapy core-needle biopsy and post-therapy surgical excision specimens. The following factors were considered pre- and post-chemotherapy for their relationship with relapse-free and overall survival: age, menstrual status, T and N stage, pre-therapy operability, Ki67, ER, PgR, HER2, grade, histological subtype, vascular invasion, clinical response, chemotherapy regimen, type of surgery performed, adjuvant therapy, pathological tumour size and nodal involvement. *Results* In a matched cohort of 103 patients, on multivariate analysis of relapse-free survival, post-therapy Ki67 was the only significant independent prognostic factor. On multivariate analysis for overall survival, both pre- and excision Ki67 were significant independent predictors but the latter showed a stronger prognostic impact. The highest and lowest tertiles of excision Ki67 had different prognosis for both 5-year relapse-free (27% vs. 77%) and overall (39% and 93%) survival. In a cohort of 284 patients with only excision samples, post-therapy Ki67 was a significant independent prognostic factor on multivariate analysis. *Conclusion* Post-chemotherapy Ki67 is a strong predictor of outcome for patients not achieving a pathological complete response.

Keywords Breast cancer · Neoadjuvant chemotherapy · Post-therapy proliferation

R. L. Jones · J. Salter · M. Dowsett (✉)
Academic Department of Biochemistry, Royal Marsden Hospital, Fulham Road, London SW3 6JJ, UK
e-mail: mitch.dowsett@icr.ac.uk

R. A'Hern
Institute of Cancer Research Clinical Trials and Statistics Unit, Sutton, Surrey, UK

A. Nerurkar · M. Parton · I. E. Smith
Breast Unit, Royal Marsden Hospital, London, UK

J. S. Reis-Filho
Breakthrough Breast Cancer Research Centre, Institute of Cancer Research, London, UK

Introduction

Neoadjuvant chemotherapy is well established in the treatment of large potentially operable and locally advanced breast cancer [1]. Administration of chemotherapy in this way achieves a clinical response in 60–90% of patients with invasive breast cancer. Consequently, downstaging of tumours can occur allowing either mastectomy in those initially deemed inoperable or breast conserving surgery in those originally only suitable for mastectomy [1]. Pathological complete response (pCR) following neoadjuvant chemotherapy, which is seen in 3–26% of patients, is a good but not perfect predictor of survival.

It is well documented that higher levels of the proliferation marker Ki67 are associated with poorer survival in breast cancer [2, 3]. Ki67 detects proliferating cells in G1, S, G2 and mitosis, but not in the resting phase G0 [4]. The monoclonal antibody MIB-1, has allowed the assessment of Ki67 in formalin-fixed tissue sections and has shown good correlation with the original Ki67 antibody [5]. MIB-1 has subsequently been widely adopted as a marker of proliferation [2].

A number of studies have addressed the predictive and prognostic significance of pre-neoadjuvant chemotherapy Ki67. Although these studies have produced partially conflicting results, it is apparent that higher pre-therapy proliferation is associated with a better response and that a significant fall in Ki67 occurs following neoadjuvant chemotherapy [6, 7]. We have previously shown for neoadjuvant endocrine therapy, that Ki67 assessment performed in samples already exposed to therapy more accurately predicts outcome than its assessment in pre-therapy biopsies, but this is not known for chemotherapy [8]. We rationalised that this may be due to the on-treatment Ki67 acting to integrate 2 pieces of information on outcome: (a) the intrinsic prognostic importance of baseline Ki67 and (b) the benefit-related change in Ki67 resulting from treatment. We hypothesised that this relationship might extend to chemotherapy. Therefore, the aim of this study was to compare the prognostic significance of Ki67 when assessed alongside other known markers of prognosis and prediction both before and after neoadjuvant chemotherapy with or without endocrine therapy.

Patients and methods

Clinical methodology

A retrospective analysis of a prospectively maintained clinical database was performed to identify patients treated with neoadjuvant chemotherapy for operable or locally advanced breast cancer between 1985 and 2005. During this period a number of patients were treated with concurrent neoadjuvant endocrine therapy. Patients who were ER negative (in the pre-therapy biopsy specimen) and were treated with neoadjuvant tamoxifen were classified as having received chemotherapy alone, as tamoxifen would not be expected to have significant activity in such cases.

Full blood count, standard serum biochemistry, chest X-ray and further investigations (if indicated) were performed to exclude metastatic disease. Neoadjuvant chemotherapy regimens included anthracycline-based schedules incorporating epirubicin 60 mg/m^2 or doxorubicin 60 mg/m^2. Mitoxantrone-based regimens and CMF (cyclophosphamide, methotrexate and 5-FU) were also used over this time period. Six cycles of chemotherapy were planned, although some of the infusional regimens involved 8 cycles of treatment. Breast conserving surgery (BCS) was performed by macroscopic excision of the residual tumour with a surrounding margin of normal tissue. All women treated with BCS received adjuvant radiotherapy. Radiotherapy was administered to mastectomy patients with axillary node involvement. All patients received adjuvant tamoxifen 20 mg per day, unless contraindicated. From 1997 onwards, oestrogen (ER) and progesterone (PgR) receptor negative patients did not receive adjuvant tamoxifen.

Patients were followed-up every 3 months for the first 2 years and then 6 monthly until 5 years. Subsequently yearly clinical and mammographic follow-up was performed.

Tissue acquisition

Core biopsies were obtained following local anaesthetic infiltration of the skin using a 14-gauge needle on a spring-loaded device and surgical samples were obtained at routine resection. Excision specimens were obtained from a representative section of the surgical specimen. The tissues were formalin-fixed and paraffin-embedded, and subsequently cut into 4 μm sections and mounted onto slides. Haematoxylin and eosin staining was performed. At the time of treatment histological type and grade was assessed by a specialist breast pathologist [9]. Specimens were not re-graded for the purpose of this study.

The first cohort consisted of 103 patients treated with neoadjuvant chemoendocrine or chemotherapy with matched pre- and post-therapy tissue available (designated the matched cohort). The second cohort consisted of 284 patients with surgical excision tissue available treated with neoadjuvant chemotherapy alone irrespective of the availability of a diagnostic core biopsy (excision cohort). The excision cohort therefore included the matched cohort. Consequently, none of the patients included in this study attained a pCR or near pCR.

Immunohistochemistry

For Ki67 staining, 4 μm sections were dewaxed in xylene and then hydrated by means of a series of graded ethanol baths and rinsed in water. Endogenous peroxidase activity was blocked. Antigen retrieval was performed by microwaving at full power (750 W) in citrate buffer pH 6.0 for 10 min. MIB-1 primary antibody (Dako, Denmark) was used at a dilution of 1:50, and incubated for an hour at room temperature. All washes and dilutions were performed with phosphate-buffered saline (PBS). Biotinylated rabbit anti-mouse immunoglobulin was applied followed by avidin-biotin complex (ABC) (Dako), peroxidase activity was developed with diaminobenzene (DAB) (Sigma, USA) and counterstaining conducted with haematoxylin. Stained sections were examined using a standard light microscope ×40 objective using a 10 × 10 eye-piece graticule, with the observer blinded to patient outcome. Ki67 score was defined as the percentage of total number of tumour cells (at least 1000) with nuclear staining over 10 high powered fields (×40).

The same staining procedure as that described for MIB-1 was used for ER, with microwave antigen retrieval.

The primary antibody used 6F11 (Novocastra, UK) was incubated at a dilution of 1:40 for 2 h at room temperature. ER was evaluated using a Histo-score (H-score), which incorporates evaluation of intensity of stain (0–3) and number of cells staining (range of score 0–300).

Statistical analysis

In the matched cohort the following factors were considered pre-therapy and at excision (where relevant) for their relationship with relapse-free (RFS) and overall (OS) survival: age, menstrual status, T and N stage, pre-therapy operability, Ki67, ER, grade, histological subtype, vascular invasion, clinical response, chemotherapy regimen, type of surgery performed, adjuvant therapy, pathological tumour size, nodal involvement and if available HER2 and PgR status.

In the excision cohort, age, menstrual status, clinical T and N stage and excision factors were evaluated for their relationship with long-term outcome.

Associations between two variables were assessed as follows: nominal tabulated data were analysed using the chi-squared test (for 2×2 contingency tables Fisher's exact test was used); if one factor was ordinal the Kruskal Wallis test was used if more than 2 groups were being compared, the Mann Whitney test for trend being employed to compare two groups. If two ordinal factors were being assessed Spearman Rank Correlation was employed. Univariate and multivariate survival analysis of RFS and OS was carried out using Cox regression. RFS was defined as the time from the date of presentation to the date of first local relapse, distant relapse or occurrence of a new primary tumour. Patients without an event were censored at the time of last follow-up. OS was defined as the time from presentation to death. All P values were two tailed and 95% confidence intervals were adopted.

Multivariate analysis was performed in a forward stepwise fashion, the most significant additional variable (satisfying $P < 0.05$) being added at each stage, cases with missing values for any of the variables in the model were excluded from analysis. Ninety-five percent confidence intervals were used to express ranges within which true parameter values were likely to lie.

Ki67 was measured as a continuous score which is typically positively skewed. Analysis was undertaken by log transforming Ki67 and using log(Ki67) as a covariate to investigate whether there is a linear increase in the probability of relapse with increasing Ki67 value. The trend in hazards will not necessarily be linear, therefore quadratic and cubic centred components were also considered to allow the pattern of hazards with increasing Ki67 value to vary from a simple linear trend.

It should be noted that if both excision and biopsy Ki67 are statistically significant that implies the proportional change is important because, if $\beta 1$ and $\beta 2$ are the Cox coefficients,

$$\begin{aligned}&\beta_1 \ln(\mathrm{Ki67}_{\mathrm{excis}}) + \beta_2 \ln\left(\mathrm{Ki67}_{\mathrm{biop}}\right)\\ &= \beta_1 \ln(\mathrm{Ki67}_{\mathrm{excis}}) - \beta_1 \ln\left(\mathrm{Ki67}_{\mathrm{biop}}\right) + \beta_1 \ln\left(\mathrm{Ki67}_{\mathrm{biop}}\right)\\ &\quad + \beta_2 \ln\left(\mathrm{Ki67}_{\mathrm{biop}}\right)\\ &= \beta_1 \ln\left(\mathrm{Ki67}_{\mathrm{excis}}/\mathrm{Ki67}_{\mathrm{biop}}\right) + (\beta_1 + \beta_2) \ln\left(\mathrm{Ki67}_{\mathrm{biop}}\right)\end{aligned}$$

So the hazard ratio due to biopsy and excision Ki67 values can be re-written in terms of the proportional change and the biopsy value. This also demonstrates that if $\beta_1 = -\beta_2$ then only the proportional change will be important.

To illustrate the combined effect of biopsy and excision Ki67 in the matched patients a prognostic index was derived.

The sum of the Cox regression coefficients of the linear and quadratic excision Ki67 components multiplied by the transformed biopsy values were used to create a prognostic score for each patient. The quartiles of all the prognostic scores were then used to categorise the patients into four groups to display outcome.

Results

Matched cohort

One hundred and three patients with matched pre- and post-therapy (excision) specimens were available for analysis. In this cohort, 61 women were treated with neoadjuvant chemotherapy alone and 42 received concurrent neoadjuvant chemoendocrine therapy. The clinical characteristics of this cohort of patients are displayed in Table 1. Of the 61 women treated with chemotherapy alone, 54 (88.5%) received anthracycline-based regimens and 7 (11.5%) non-anthracycline schedules. In the subgroup of 42 treated with chemoendocrine therapy, 40 (95.2%) received anthracycline-based regimens and 2 (4.8%) non-anthracycline schedules.

Relapse-free survival

On univariate analysis pre-neoadjuvant systemic therapy factors with a significant inverse relationship with RFS were; higher clinical T ($P < 0.001$), higher N stage ($P = 0.002$), higher Ki67 ($P < 0.001$ for both linear and quadratic components) and ER negativity ($P = 0.003$) (Table 2). Post-therapy factors with a significant inverse relationship with RFS included higher Ki67 ($P < 0.001$ for both linear and quadratic components), higher tumour grade ($P = 0.01$), larger pathological tumour size ($P = 0.02$), higher lymph node involvement ($P < 0.001$)

Table 1 Clinical characteristics of both cohorts

	Matched cohort		Excision cohort	
	Chemotherapy	Chemoendocrine	Chemotherapy	Chemoendocrine
Age	49 (32–75)	47 (29–64)	49 (29–75)	49 (29–64)
Menstrual status				
Pre	35 (57.4%)	25 (59.5%)	113 (56.2%)	45 (54.2%)
Peri	5 (8.2%)	6 (14.3%)	24 (11.9%)	14 (16.9%)
Post	17 (27.9%)	8 (19.0%)	52 (25.9%)	20 (24.1%)
Hysterectomy	4 (6.6%)	3 (7.1%)	11 (5.5%)	4 (4.8%)
Male	0	0	1 (0.5%)	0
Tumour size				
T1	1 (1.6%)	0	2 (1.0%)	2 (2.4%)
T2	26 (42.6%)	18 (42.9%)	91 (45.3%)	39 (47.0%)
T3	26 (42.6%)	23 (54.8%)	77 (38.3%)	35 (42.2%)
T4	8 (13.1%)	1 (2.4%)	30 (14.9%)	7 (8.4%)
Not available	0	0	1 (0.5%)	0
Nodal involvement				
N0	31 (50.8%)	21 (50.0%)	100 (49.8%)	42 (50.6%)
N1	29 (47.5%)	21 (50.0%)	95 (47.3%)	37 (44.6%)
N2	1 (1.6%)	0	5 (2.5%)	3 (3.6%)
N3	0	0	1 (0.5%)	1 (1.2%)
Initial operability				
Operable	55 (90.2%)	40 (95.2%)	177 (88.1%)	75 (90.4%)
Locally advanced	6 (9.8%)	2 (4.8%)	24 (11.9%)	8 (9.6%)
ER status				
Positive	37 (60.7%)	38 (90.5%)	88 (43.8%)	61 (73.5%)
Negative	23 (37.7%)	0	64 (31.8%)	0
Not known	1 (1.6%)	4 (9.5%)	49 (24.4%)	22 (26.5%)
Treatment regimen				
Anthracycline-based	54 (88.5%)	40 (95.2%)	163 (81.1%)	69 (83.1%)
Non-anthracycline	7 (11.5%)	2 (4.8%)	38 (18.9%)	14 (16.9%)

and no adjuvant endocrine therapy ($P < 0.001$). These results are displayed in Table 2.

On multivariate analysis, only excision Ki67 score ($P < 0.001$ for both linear and quadratic components) was found to be a significant independent predictor of RFS in this cohort. Ki67 scores derived from diagnostic biopsies were not significant, but a trend was observed as the hazard ratio for the linear component was 1.6 ($P = 0.10$) and for the quadratic component it was 1.1 ($P = 0.06$).

Figure 1 displays RFS by biopsy and excision Ki67 tertiles respectively for the 103 patients. The importance of the quadratic component is illustrated in this Figure since the increase hazard can be seen to not increase uniformly with Ki67 value but is confined to higher values. After 5 years the highest and lowest tertiles of excision Ki67 had different prognosis, RFS 27% and 77%; OS 39% and 93%, respectively.

Figure 2 displays RFS by a prognostic index derived by averaging the hazard ratios for each component of biopsy and excision Ki67 tertiles for the 103 patients.

Overall survival

On univariate analysis the following pre-neoadjuvant systemic therapy factors had a significant inverse relationship with OS; higher clinical T stage ($P < 0.001$), higher Ki67 ($P < 0.001$ for both linear and quadratic components) and ER negativity ($P = 0.006$) (Table 3). Post-neoadjuvant systemic therapy factors with a significant inverse relationship with OS on univariate analysis were; higher Ki67 ($P < 0.001$ for linear and $P = 0.003$ for quadratic components respectively), ER negativity ($P = 0.04$), higher tumour grade ($P = 0.04$), pathological tumour size ($P = 0.005$), lymph node involvement ($P = 0.03$) and no adjuvant endocrine therapy ($P = 0.001$). On multivariate analysis, excision Ki67 score was found to be a significant independent predictor of OS ($P < 0.001$ for linear and

Table 2 Univariate and multivariate analysis of relapse-free survival for the matched cohort of 103 patients

Factor	Univariate analysis		Multivariate analysis	
	P value	HR (95%CI)	*P* value	HR (95% CI)
Age	NS (0.9)	1.0 (0.7–1.5)	NS	
Menstrual status	NS (0.9)		NS	
Pre		1.0		
Peri		1.1 (0.5–2.8)		
Post		1.2 (0.6–2.2)		
T stage	<0.001		NS	
T1				
T2		1.0		
T3		1.8 (0.95–3.4)		
T4		2.2 (0.9–5.6)		
N stage	0.002		NS	
N0		1		
N1		1.2 (0.7–2.0)		
N2		5.1 (0.7–39.1)		
Operability	NS (0.2)		NS	
Operable		1.0		
Locally advanced		1.7 (0.7–4.1)		
Pre-therapy Ki67			NS	
Linear	<0.001	2.4 (1.5–3.9)		
Quadratic	<0.001	1.2 (1.1–1.4)		
Pre-therapy ER status	0.003		NS	
Negative		1.0		
Positive		0.4 (0.2–0.7)		
Pre-therapy PgR status	NS (0.4)		NS	
Negative		1.0		
Positive		0.03 (0–64.1)		
Pre-therapy HER2 status	NS (1.0)		NS	
Negative		1.0		
Positive		1.0 (0.3–2.9)		
Pre-therapy grade	NS (0.2)		NS	
1		1.0		
2		0.4 (0.1–2.0)		
3		1.0 (0.2–4.4)		
Pre-therapy histology	NS (0.5)		NS	
IDC		1.0		
ILC		0.8 (0.3–1.7)		
Chemoendocrine therapy	0.01		NS	
No		1.0		
Yes		0.6 (0.4–0.9)		
Anthracycline	NS (0.8)		NS	
No		1.0		
Yes		0.9 (0.4–2.2)		
Response to first line neoadjuvant therapy	NS (0.5)		NS	
CR		1.0		
PR		0.8 (0.4–1.8)		
SD		0.8 (0.3–2.1)		
PD		1.5 (0.5–4.1)		

Table 2 continued

Factor	Univariate analysis		Multivariate analysis	
	P value	HR (95%CI)	*P* value	HR (95% CI)
Excision Ki67				
Linear	<0.001	1.9 (1.6–2.3)	<0.001	1.9 (1.6–2.3)
Quadratic	<0.001	1.2 (1.1–1.3)	<0.001	1.2 (1.1–1.3)
Excision ER status	NS (0.1)		NS	
Negative		1.0		
Positive		0.6 (0.3–1.1)		
Excision PgR status	NS (0.1)		NS	
Negative		1.0		
Positive		0.2 (0–1.6)		
Excision HER2 status	NS (0.9)		NS	
Negative		1.0		
Positive		0.9 (0.3–3.1)		
Excision grade	0.01		NS	
1		1.0		
2		2.3 (0.7–7.9)		
3		3.9 (1.1–13.1)		
Excision histology	NS (0.7)		NS	
IDC		1.0		
ILC		0.9 (0.5–1.6)		
Vascular invasion	NS (0.4)		NS	
Absent		1.0		
Present		1.3 (0.7–2.4)		
Pathological tumour size	0.02	2 (1.1–3.6)	NS	
Pathological LN status	<0.001		NS	
0		1.0		
1–3		1.5 (0.7–3.1)		
4+		2.1 (1.0–4.7)		
Type of surgery performed	NS (0.4)		NS	
BCS		1.0		
Mastectomy		1.3 (0.7–2.2)		
Adjuvant endocrine therapy	<0.001		NS	
No		1.0		
Yes		0.2 (0.1–0.5)		
Adjuvant chemotherapy	NS (1.0)		NS	
No		1.0		
Yes		1.0 (0.2–4.4)		

$P = 0.007$ for quadratic components respectively) as was pre-therapy Ki67 ($P = 0.02$ for both linear and quadratic components) (Table 3). Figure 2 displays OS by biopsy and excision Ki67 tertiles respectively for the 103 patients.

Excision cohort

A total of 284 patients with surgical excision specimens available were treated with either neoadjuvant chemo- or chemoendocrine therapy, of these 201 were treated with chemotherapy alone. One hundred and seventy seven (88.1%) patients of those receiving chemotherapy alone and 75 (90.4%) of those receiving chemoendocrine therapy had breast tumours that were potentially operable before neoadjuvant systemic therapy. Ninety-one (45.3%) patients in the chemotherapy alone subgroup had T2 tumours and 39 (47.0%) in the subgroup treated with neoadjuvant chemoendocrine therapy. Most patients in both subgroups had either no clinical lymph node involvement (49.8% and 50.6%, respectively) or N1 disease (47.3% and 44.6%, respectively).

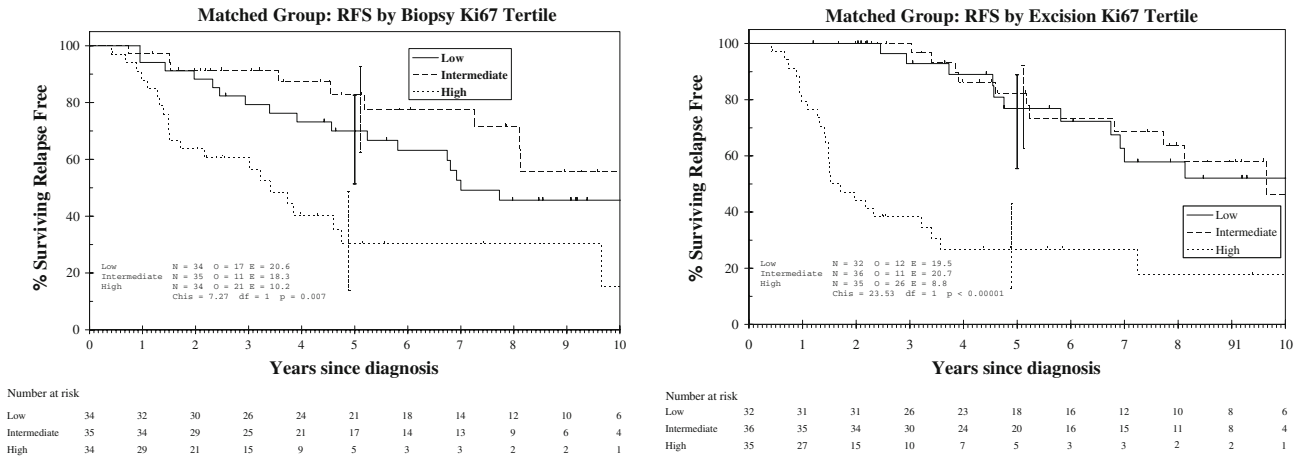

Fig. 1 Kaplan Meier curve displaying relapse-free survival by biopsy and excision Ki67 tertiles in the matched cohort of 103 patients (**a**) Matched group: RFS by biopsy Ki67 tertile. (**b**) Matched Group: RFS by excision Ki67 tertile

Relapse-free survival

On univariate analysis the following factors were found to have a significant inverse relationship with RFS: younger age ($P = 0.01$), higher T stage ($P < 0.001$), higher N stage ($P < 0.001$), patients with locally advanced disease compared to those with operable tumours at presentation ($P < 0.001$), excision Ki67 ($P < 0.001$), ER negative ($P < 0.001$), PgR negative ($P = 0.04$), higher tumour grade ($P < 0.001$), vascular invasion ($P = 0.001$), pathological tumour size ($P = 0.003$), pathological lymph node involvement ($P < 0.001$) and no adjuvant endocrine therapy in patients with ER positive tumours ($P < 0.001$).

On multivariate analysis of all factors associated with RFS on univariate analysis the following had significant independent value; pre-therapy T ($P = 0.002$) and N stage ($P = 0.002$), excision Ki67 ($P < 0.001$), excision pathological lymph node involvement ($P < 0.001$) and no adjuvant endocrine therapy in patients with ER positive tumours ($P < 0.001$) (Table 4).

Overall survival

On univariate analysis the following factors were found to have a significant inverse relationship with OS; higher T stage ($P < 0.001$) and N stage ($P < 0.001$), locally advanced disease ($P = 0.002$), excision Ki67 ($P < 0.001$ for both linear and quadratic components), ER negative ($P < 0.001$), clinical progressive disease on neoadjuvant systemic therapy ($P = 0.04$), higher tumour grade ($P < 0.001$), presence of vascular invasion ($P = 0.009$), pathological tumour size ($P < 0.001$), pathological lymph node involvement ($P < 0.001$), mastectomy compared to breast conserving surgery ($P = 0.04$) and no adjuvant endocrine therapy ($P < 0.001$).

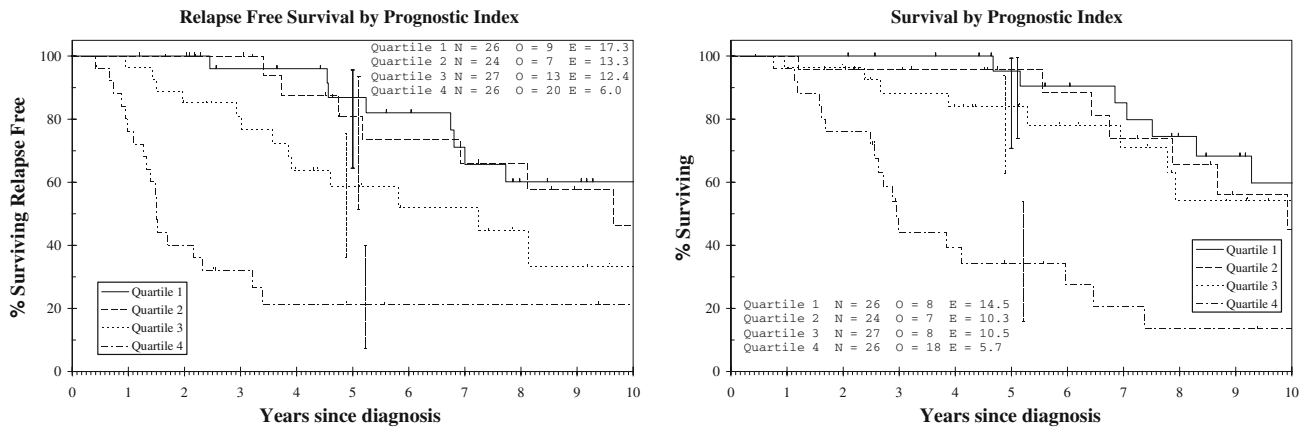

Fig. 2 Kaplan Meier curves displaying relapse-free and overall survival by prognostic index quartiles (**a**) Relapse-free survival by prognostic index. (**b**) Overall survival by prognostic index

Table 3 Univariate and multivariate analyses for overall survival in the matched cohort of 103 patients

Factor	Univariate analysis		Multivariate analysis	
	P value	HR (95% CI)	*P* value	HR (95% CI)
Age	NS (0.8)	1.0 (0.7–1.5)	NS	
Menstrual status	NS (0.5)		NS	
Pre		1.0		
Peri		0.9 (0.3–2.8)		
Post		1.5 (0.8–2.9)		
T stage	<0.001		NS	
T1				
T2		1.0		
T3		0.6 (0.2–1.4)		
T4		0.7 (0.3–1.5)		
N stage	NS (0.5)		NS	
N0		1.0		
N1		1.1 (0.6–2.1)		
N2		4.6 (0.6–35.2)		
Operability	NS (0.4)		NS	
Operable		1.0		
LA		1.4 (0.6–3.7)		
Pre-therapy Ki67				
Linear	<0.001	2.8 (1.6–4.7)	0.02	2.0 (1.1–3.4)
Quadratic	<0.001	1.2 (1.1–1.4)	0.02	1.2 (1.0–1.3)
Pre-therapy ER status	0.006		NS	
Negative		1.0		
Positive		0.4 (0.2–0.8)		
Pre-therapy PgR status	NS (0.4)		NS	
Negative		1.0		
Positive		0.03 (0–183.7)		
Pre-therapy HER2 status	NS (0.4)		NS	
Negative		1.0		
Positive		1.6 (0.6–4.3)		
Pre-therapy Grade	NS (0.3)		NS	
1		1.0		
2		1.2 (0.2–10.2)		
3		2.0 (0.3–15.9)		
Pre-therapy histology	NS (0.3)		NS	
IDC		1.0		
ILC		0.6 (0.2–1.5)		
Chemoendocrine therapy	0.02		NS	
No		1.0		
Yes		0.5 (0.2–1.0)		
Anthracycline	NS (0.8)		NS	
No		1.0		
Yes		1.2 (0.4–3.3)		
Response to first line neoadjuvant chemotherapy	NS (0.2)		NS	
CR		1.0		
PR		1.4 (0.5–4.0)		
SD		1.6 (0.5–5.3)		
PD		2.3 (0.7–7.8)		

Table 3 continued

Factor	Univariate analysis		Multivariate analysis	
	P value	HR (95% CI)	*P* value	HR (95% CI)
Excision Ki67		1.6 (1.2–2.0)		
Linear	<0.001		<0.001	1.6 (1.3–2.0)
Quadratic	0.003		0.007	1.1 (1.0–1.3)
Excision ER status	0.04		NS	
Negative		1.0		
Positive		0.5 (0.3–1.0)		
Excision PgR status	NS (0.4)		NS	
Negative		1.0		
Positive		0.02 (0–95.5)		
Excision HER2 status	NS (0.6)		NS	
Negative		1.0		
Positive		0.7 (0.2–2.5)		
Excision grade	0.04		NS	
1		1		
2		1.8 (0.5–6.0)		
3		2.9 (0.9–10.0)		
Excision histology	NS (0.2)		NS	
IDC		1.0		
ILC		0.6 (0.2–1.4)		
Vascular invasion	NS (0.3)		NS	
Absent		1.0		
Present		1.4 (0.7–2.8)		
Pathological tumour size	0.005	2.4 (1.3–4.5)	NS	
Pathological LN status	0.03		NS	
0		1.0		
1–3		1.5 (0.6–3.4)		
>4		2.6 (1.1–6.1)		
Type of surgery performed	NS (0.08)		NS	
BCS		1.0		
Mastectomy		1.7 (0.9–3.3)		
Adjuvant endocrine therapy	0.001		NS	
No		1.0		
Yes		0.2 (0.1–0.5)		
Adjuvant chemotherapy	NS (0.4)		NS	
No		1.0		
Yes		1.8 (0.4–7.6)		

A trend for survival was observed in patients with PgR negative ($P = 0.06$) disease, and women treated with neoadjuvant chemotherapy alone ($P = 0.07$).

On multivariate analysis, the following factors were independent significant factors of OS: clinical N stage ($P < 0.001$), post-therapy Ki67 ($P < 0.001$ for both linear and quadratic components) and ER negativity ($P = 0.002$). These results are displayed in Table 5.

Change in Ki67

Three models were pertinent to the role of pre-therapy and excision Ki67. If only Ki67 change alone was considered then both a linear and quadratic component were significant with an overall chi squared value of 26.7 on 2 degrees of freedom. The chi squared value is a measure of the prognostic performance of the model, the larger the value the better the performance. If only excision values were

Table 4 Univariate and multivariate analyses for relapse-free survival in the excision cohort of 284 patients

Factor	Univariate analysis		Multivariate analysis	
	P value	HR (95% CI)	*P* value	HR (95% CI)
Age	0.01	1.2 (1.0–1.4)	NS	
Menstrual status	NS (0.3)		NS	
Pre		1		
Peri		1.1 (0.6–1.8)		
Post		1.3 (0.9–1.9)		
T stage	<0.001		0.002	1.5 (1.2–1.9)
T1		1		
T2		1.9 (0.3–13.9)		
T3		3.6 (0.5–26.1)		
T4		5.3 (0.7–38.8)		
N stage	<0.001		0.002	1.7 (1.2–2.3)
N0		1		
N1		1.8 (1.3–2.6)		
N2		7.0 (3.3–15.0)		
N3		8.2 (2.0–34.0)		
Operability	<0.001		NS	
Operable		1		
Locally advanced		2.3 (1.5–3.7)		
Excision Ki67	<0.001	1.3 (1.2–1.5)	<0.001	1.3 (1.1–1.5)
ER status	<0.001		NS	
Negative		1		
Positive		0.4 (0.3–0.6)		
PgR status	0.04		NS	
Negative		1		
Positive		0.3 (0.1–0.9)		
HER2 status	NS (0.1)		NS	
Negative		1		
Positive		1.5 (0.9–2.8)		
Grade	<0.001		NS	
1		1		
2		2.7 (1.1–6.7)		
3		5.2 (2.1–12.9)		
Histology	NS (0.3)			
IDC		1		
ILC		0.8 (0.5–1.2)		
Chemoendocrine therapy	NS (0.1)		NS	
No		1		
Yes		0.8 (0.5–1.1)		
Anthracycline therapy	NS (0.2)			
No		1		
Yes		1.3 (0.9–2.0)		
Response to neoadjuvant therapy	NS (0.3)		NS	
CR		1		
PR		0.9 (0.6–1.6)		
SD		1.1 (0.6–2.0)		
PD		1.4 (0.7–3.0)		

Table 4 continued

Factor	Univariate analysis		Multivariate analysis	
	P value	HR (95% CI)	*P* value	HR (95% CI)
Vascular invasion	<0.001		NS	
Not present		1		
Present		1.9 (1.3–2.7)		
Pathological tumour size	0.003	1.6 (1.2–2.1)	NS	
Pathological LN status	<0.001		<0.001	1.7 (1.3–2.1)
0		1		
1–3		1.6 (1.0–2.5)		
>4		3.0 (1.9–4.7)		
Type of surgery performed	NS (0.2)		NS	
BCS		1		
Mastectomy		1.3 (0.9–1.8)		
Adjuvant endocrine therapy	<0.001		<0.001	0.4 (0.3–0.6)
No		1.0		
Yes		0.4 (0.3–0.6)		
Adjuvant Chemotherapy	NS (0.4)			
No		1		
Yes		0.8 (0.4–1.4)		

considered then a linear and quadratic component were also significant with an overall chi squared value of 51.1 on 2 degrees of freedom. This model was highly significantly better than the previous (change in Ki67) model. The model with the highest chi squared value was composed of both biopsy and excision values, this had an overall chi squared value of 57.0 on 4 degrees of freedom and was not a significant improvement on the former model but was suggestive of an improved performance when both pre-therapy and excision values were used ($0.05 < P < 0.10$).

Discussion

This study in a cohort of patients with matched pre- and post-treatment samples indicates that Ki67 following neo-adjuvant systemic therapy is a strong predictor of long-term outcome (Fig. 3). Given the need for invasive disease in the excision sample for a patient to be included in this study, we specifically addressed the impact of Ki67 labelling indices on the survival of patients who received neoadjuvant chemotherapy and did not achieve pCR. The greater significance of Ki67 in the excision sample than the diagnostic sample may be due to this identifying patients in whom there remains a high chance of residual highly proliferative micrometastatic disease after neoadjuvant chemoendocrine or chemotherapy. The suggestion that both biopsy Ki67 and excision Ki67 are important in the matched cohort implies both the baseline Ki67 and the proportional change may be important predictors of outcome (see statistical analysis section). The effect of these two predictors considered together can be illustrated by calculating a combined prognostic index (Fig. 2), though clearly this is an exploratory analysis. Our current study, although retrospective, is strengthened by the analysis of a relatively large paired cohort of patients and the use of a well established methodology. In addition as this was a single centre study standardised therapeutic approaches were employed.

Table 6 displays the results of other studies assessing the prognostic value of post-neoadjuvant chemotherapy and change in Ki67. Post-therapy Ki67 was found to be the only significant independent factor associated with overall survival, on multivariate analysis, in a series 48 patients with locally advanced breast cancer treated within the context of a phase II trial [10]. Two other studies have found pre- to post-therapy change in Ki67 to be a significant independent predictor of disease-free and relapse-free survival [11 and 12 respectively]. Osborne et al. studied median pre- and post-therapy Ki67 in 25 relapsed and 33 recurrence-free basal-like breast cancers. An increase in median Ki67 following therapy was observed in the relapsed subgroup and a decrease in the subgroup that did not relapse. The difference in post neoadjuvant chemotherapy Ki67 and the change in Ki67 score were found to be statistically significant between the two groups [13].

In contrast, others have not found an independent relationship between post-neoadjuvant chemotherapy Ki67 and survival [14–18]. Three studies found ER status to be the sole independent predictor of DFS or RFS on multivariate

Table 5 Univariate and multivariate analyses for overall survival in the excision cohort of 284 patients

Factors	Univariate analysis		Multivariate analysis	
	P value	HR (95% CI)	*P* value	HR (95% CI)
Age	0.8	1.0 (0.8–1.3)	NS	
Menstrual status	NS (0.2)		NS	
Pre		1		
Peri		1.1 (0.6–1.9)		
Post		1.4 (1.0–2.2)		
T stage	<0.001		NS	
T1				
T2		1		
T3		1.5 (1–2.3)		
T4		2.3 (1.4–3.9)		
N stage	<0.001		<0.001	
N0		1		
N1		1.8 (1.2–2.6)		
N2		6.5 (2.7–15.4)		
N3		5.8 (0.8–42.6)		
Operability	0.002		NS	
Operable		1		
Locally advanced		2.2 (1.3–3.7)		
Excision Ki67	<0.001	1.4 (1.2–1.6)	<0.001	
ER status	<0.001		0.002	
Negative		1		
Positive		0.4 (0.3–0.6)		
PgR status	NS (0.06)		NS	
Negative		1		
Positive		0.3 (0.1–1.1)		
HER2 status	NS (0.8)		NS	
Negative		1		
Positive		1.1 (0.5–2.2)		
Grade	<0.001		NS	
1		1		
2		2.7 (1.0–7.6)		
3		4.7 (1.7–13.1)		
Histology	NS (0.1)		NS	
IDC		1		
ILC		0.6 (0.4–1.1)		
Chemoendocrine therapy	NS (0.07)		NS	
No		1		
Yes		0.7 (0.5–1.0)		
Anthracycline	NS (0.3)		NS	
No		1		
Yes		1.3 (0.8–2.0)		
Response to neoadjuvant therapy	0.04		NS	
CR		1		
PR		1.2 (0.6–2.3)		
SD		1.6 (0.8–3.5)		
PD		2.1 (0.9–4.9)		

Table 5 continued

Factors	Univariate analysis		Multivariate analysis	
	P value	HR (95% CI)	*P* value	HR (95% CI)
Vascular invasion	0.009		NS	
Not present		1		
Present		1.7 (1.2–2.6)		
Pathological tumour size	<0.001	1.9 (1.3–2.7)	NS	
Pathological LN status	<0.001		NS	
0		1		
1–3		1.9 (1.1–3.3)		
>4		3.5 (2.1–5.9)		
Type of surgery performed	0.04		NS	
BCS		1		
Mastectomy		1.5 (1.0–2.2)		
Adjuvant endocrine therapy	<0.001		NS	
No		1		
Yes		0.4 (0.3–0.6)		
Adjuvant chemotherapy	NS (0.5)		NS	
No		1		
Yes		0.8 (0.4–1.5)		

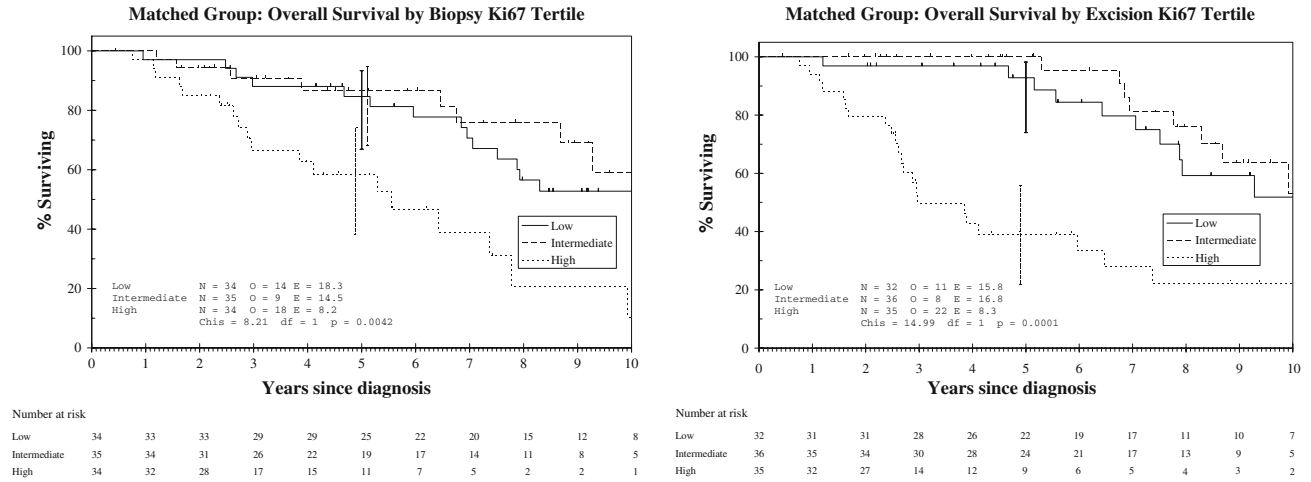

Fig. 3 Kaplan Meier curve displaying overall survival by excision Ki67 tertile in the matched cohort of 103 patients (**a**) Matched group: Overall survival by Ki67 biopsy tertile. (**b**) Matched group: Overall Survival by Ki67 excision tertile

analysis [14, 16, 19]. Bottini et al. observed on multivariate analysis that clinical response, tumour size and PgR status were independent predictors for disease recurrence [15, 20]. Faneyte and colleagues assessed the role of Ki67, ER, HER2, p53 and bcl2 in 97 women with breast cancer and extensive axillary node involvement and found none of these factors to be associated with DFS and OS [17].

The current findings have similarities to those we observed in patients treated with endocrine therapy. The IMPACT (Immediate Preoperative Anastrozole, Tamoxifen or Combined with Tamoxifen) trial has demonstrated that higher Ki67 levels predict worse recurrence-free survival better following just 2 weeks of neoadjuvant endocrine therapy compared to the baseline pre-therapy Ki67 [8]. It remains to be determined whether measuring the level of Ki67 after a short period of chemotherapy, e.g. after a single course of therapy, will provide as much additional prognostic value as at excision.

Our data are not consistent with the hypothesis that residual malignant disease after chemotherapy is enriched by a stem-like cell population with low proliferation that is associated with worse outcome [23]. However, a subpopulation of such stem-like cells may reside in the otherwise highly proliferative residual disease.

The results of our study suggest that Ki67 after neoadjuvant chemoendocrine or chemotherapy alone is a strong

Table 6 Studies assessing the prognostic role of pre-, post- and change in proliferation and neoadjuvant chemotherapy

Author (Year) [Reference]	Stage Number Biopsy or FNA	Regimen Follow-up	Biomarkers	Analysis of overall survival p value (95%CI) of only the significant biomarkers shown	Analyses of RFS, DFS, PFS or DFI p value (95%CI) of only the significant biomarkers shown
Daidone et al (1999) [19]	>T2 231 Biopsy	CMF FAC, FEC or FNC Doxorubicin 99 (3-122) months	PRE + POST TLI ER (-) PgR (-) bcl2, p53 + bax DNA ploidy	NOT DONE	Univariate, RFS 0.0007 (1.2-2.8) 0.075 (1.0-2.2)
Colleoni et al (2004) [14]	T2-3 N0-2 399 Biopsy	AC ECF FLN V-FUP AT/ET 3.8 years	PRE + POST Ki67 HR, ER (+) Grade HER2 Change Ki67 LN status	NOT DONE	Multivariate, DFS ER(+), NO p value
Schneeweiss et al (2004) [18]	Stage I-III 240 Biopsy	Anthracycline-based 6.4 years (1-10.4 years)	(PRE) + POST Ki67 HR Grade, lower bcl2 + p53 Size LN status (-) Clinical Response	Multivariate <0.001 (1.5-4.1) 0.005 (1.1-2.1) <0.001 (1.5-3.1)	Multivariate, DDFS 0.006 (1.2-2.7) 0.02 (1.1-2.8) <0.001 (1.7-3.6)
Bottini et al (2001) [15]	T2-4N0-1 157 Biopsy	CMF-T Epirubicin 52.7 months	PRE + POST Ki67 ER PgR (+) HER2 bcl2 + p53 Change Ki67 Clinical response Treatment Menopausal status Tumour size Post LN status	NOT DONE	Multivariate, RFS PgR, p<0.02 Response, p=0.03 Size, p<0.03 NS (0.07)
Penault-Llorca et al (2003) [16]	T2-4N0-? 115 Biopsy	Anthracycline-based 63 months	PRE + POST Ki67 Pre ER (+) Post ER (+) PgR HER2 p53 Tumour size LN status Age	NOT DONE	Multivariate, DFS p=0.009 (1.3-7.1) p=0.002 (1.7-11.0)
Takada et al (2004) [11]	T1-T4 72 Biopsy Survival group = 42	FAC CEF AC EC 2.7 years	PRE + POST Ki67 ER + PgR HER2 p53 Pre M30 Post M30 Post Ki67/M30 Change Ki67 M30↑ / Ki67↓ LN status (-)	Multivariate High, p=0.0177 Low, p=0.01 p=0.0212 p=0.0459	Multivariate, DFS High, p=0.035 Low, p=0.0005 ↓, p<0.0001 p=0.0259

Table 6 continued

Billgren et al (1999) [12]	T2-3, +/- M1 51 FNA	FEC 39 (17-72) months	PRE + DAY 21 Ki67 HR LN status (-) Change Ki67		Multivariate, RFS p=0.021 ↓25%, p=0.032 (for worse RFS)
Honkoop et al (1998) [21]	IIIA – IIIB 42 Biopsy	AC (90mg/m^2 + 1000mg/m^2 respectively) 32 (10-72) months	PRE + POST Ki67 ER Cd31 Pre + post p53 Pre + post P-gp Pre P-gp/ p53 Post P-gp/ p53 pCR # chemotherapy LN status	Multivariate P=0.04 P=0.04 P=0.03	Multivariate, DFS (p=0.008) p=0.04 p=0.05 p=0.04 p=0.003 p=0.05
Faneyte et al (2003) [17]	Operable N3M0 97 Biopsy	FEC 49 (21-76)	PRE + POST Ki67 ER HER2 p53 + bcl2 Response score -pCR	Univariate No association	Univariate, DFS p=0.04
Lee et al (2007) [10]	Stage IIB-IIIC 61 Biopsy	Doxorubicin + Docetaxel 37.9 months	PRE + POST Ki67 (?1.0) Grade ER (+) PgR HER2 p53 Change Ki67 Tumour size Age Performance status Menopausal status Adjuvant therapy Inflammatory	Multivariate Post, p=0.033	Univariate, RFS NO association
Vincent-Salomon et al (2004) [22]	T2-T4N0-2 55 Biopsy	FAC 52 months	PRE + POST Ki67 SPF MI ER + PgR Age Grade pCR Clinical stage	Univariate >50%↓, p=0.02	Univariate, MFI age>40, p=0.04 NS (p=0.07)

Abbreviations: AC, doxorubicin, cyclophosphamide; AT, doxorubicin, taxotere; CMF-T, cyclophosphamide, methotrexate, 5-FU, tamoxifen; CEF, cyclophosphamide, epirubicin, 5-FU; DFS, disease-free survival; DDFS, distant disease-free survial; EC, epirubicin, cyclophosphamide; ECF, epirubicin, cyclophosphamide, 5-FU; ET, epirubicin, taxotere; FAC, 5-FU, doxorubicin, cyclophosphamide; FEC, 5-FU, epirubicin, cyclophosphamide; FLN, 5-FU, lederfolin, navelbine; FNC, 5-FU, novantrone, cyclophosphamide; FNA, fine needle aspiration; HR, hormone receptor; LN, lymph node; MFI, metastasis free interval; MI, mitotic index; RFS, relapse-free survival; SPF, S-phase fraction; V-FUP, Vinorelbine, 5-FU, (*cis*)platinum

predictor of long-term outcome and further prognostic information may also be gained from pre-treatment Ki67. The greater significance of Ki67 in the excision sample may be due to this identifying patients in whom residual highly proliferative disease remains after neoadjuvant systemic therapy. While baseline Ki67 predicts for a high chance of

pCR, in those that do not achieve a pathological remission and maintain highly proliferative disease the outcome is poor. Patients with tumours displaying elevated proliferation post-neoadjuvant systemic therapy may benefit from non-cross resistant adjuvant chemotherapy schedules.

References

1. Jones RL, Smith IE (2006) Neoadjuvant treatment for early-stage breast cancer: opportunities to assess tumour response. Lancet Oncol 7:869–874. doi:10.1016/S1470-2045(06)70906-8
2. Urruticoechea A, Smith IE, Dowsett M (2005) Proliferation marker Ki-67 in early breast cancer. J Clin Oncol 23:7212–7220. doi:10.1200/JCO.2005.07.501
3. de Azambuja E, Cardoso F, de Castro G Jr et al (2007) Ki-67 as prognostic marker in early breast cancer: a meta-analysis of published studies involving 12, 155 patients. Br J Cancer 96:1504–1513. doi:10.1038/sj.bjc.6603756
4. Gerdes J, Lemke H, Baisch H, Wacker HH, Schwab U, Stein H (1984) Cell cycle analysis of a cell proliferation-associated human nuclear antigen defined by the monoclonal antibody Ki-67. J Immunol 133:1710–1715
5. Querzoli P, Albonico G, Ferretti S et al (1996) MIB-1 proliferative activity in invasive breast cancer measured by image analysis. J Clin Pathol 49:926–930. doi:10.1136/jcp.49.11.926
6. Archer CD, Parton M, Smith IE et al (2003) Early changes in apoptosis and proliferation following primary chemotherapy for breast cancer. Br J Cancer 89:1035–1041. doi:10.1038/sj.bjc.6601173
7. Ellis PA, Smith IE, Detre S et al (1998) Reduced apoptosis and proliferation and increased Bcl-2 in residual breast cancer following preoperative chemotherapy. Breast Cancer Res Treat 48:107–116. doi:10.1023/A:1005933815809
8. Dowsett M, Smith IE, Ebbs SR et al (2007) Prognostic value of Ki67 expression after short-term presurgical endocrine therapy for primary breast cancer. J Natl Cancer Inst 99:167–170. doi:10.1093/jnci/djk020
9. Elston CW, Ellis IO (1991) Pathological prognostic factors in breast cancer I. The value of histological grade in breast cancer: experience from a large study with long-term follow-up. Histopathology 19:403–410. doi:10.1111/j.1365-2559.1991.tb00229.x
10. Lee J, Im YH, Lee SH et al (2008) Evaluation of ER and Ki-67 proliferation index as prognostic factors for survival following neoadjuvant chemotherapy with doxorubicin/docetaxel for locally advanced breast cancer. Cancer Chemother Pharmacol 61:569–577. doi:10.1007/s00280-007-0506-8
11. Takada M, Kataoka A, Toi M et al (2004) A close association between alteration in growth kinetics by neoadjuvant chemotherapy and survival outcome in primary breast cancer. Int J Oncol 25:397–405
12. Billgren AM, Rutqvist LE, Tani E, Wilking N, Fornander T, Skoog L (1999) Proliferating fraction during neoadjuvant chemotherapy of primary breast cancer in relation to objective local response and relapse-free survival. Acta Oncol 38:597–601. doi:10.1080/028418699431186
13. Osborne CR KL, Xie X-J, Ashfaq R, Bian A, Tripathy D (2006) Neoadjuvant chemotherapy for basal-like breast cancer cohort: clinical and pathological outcomes. Breast Cancer Res Treat 100(1):S53
14. Colleoni M, Viale G, Zahrieh D et al (2004) Chemotherapy is more effective in patients with breast cancer not expressing steroid hormone receptors: a study of preoperative treatment. Clin Cancer Res 10:6622–6628. doi:10.1158/1078-0432.CCR-04-0380
15. Bottini A, Berruti A, Bersiga A et al (2001) Relationship between tumour shrinkage and reduction in Ki67 expression after primary chemotherapy in human breast cancer. Br J Cancer 85:1106–1112. doi:10.1054/bjoc.2001.2048
16. Penault-Llorca F, Cayre A, Bouchet Mishellany F et al (2003) Induction chemotherapy for breast carcinoma: predictive markers and relation with outcome. Int J Oncol 22:1319–1325
17. Faneyte IF, Schrama JG, Peterse JL, Remijnse PL, Rodenhuis S, van de Vijver MJ (2003) Breast cancer response to neoadjuvant chemotherapy: predictive markers and relation with outcome. Br J Cancer 88:406–412. doi:10.1038/sj.bjc.6600749
18. Schneeweiss A, Katretchko J, Sinn HP et al (2004) Only grading has independent impact on breast cancer survival after adjustment for pathological response to preoperative chemotherapy. Anticancer Drugs 15:127–135. doi:10.1097/00001813-200402000-00005
19. Daidone MG, Veneroni S, Benini E et al (1999) Biological markers as indicators of response to primary and adjuvant chemotherapy in breast cancer. Int J Cancer 84:580–586. doi:10.1002/(SICI)1097-0215(19991222)84:6≤580::AID-IJC7≥3.0.CO;2-W
20. Bottini A, Berruti A, Bersiga A et al (1996) Effect of neoadjuvant chemotherapy on Ki67 labelling index, c-erbB-2 expression and steroid hormone receptor status in human breast tumours. Anticancer Res 16:3105–3110
21. Honkoop AH, van Diest PJ, de Jong JS et al (1998) Prognostic role of clinical, pathological and biological characteristics in patients with locally advanced breast cancer. Br J Cancer 77:621–626
22. Vincent-Salomon A, Rousseau A, Jouve M et al (2004) Proliferation markers predictive of the pathological response and disease outcome of patients with breast carcinomas treated by anthracycline-based preoperative chemotherapy. Eur J Cancer 40:1502–1508. doi:10.1016/j.ejca.2004.03.014
23. Li X, Lewis MT, Huang J et al (2008) Intrinsic resistance of tumorigenic breast cancer cells to chemotherapy. J Natl Cancer Inst 100:672–679. doi:10.1093/jnci/djn123